

FORM PTO-1390 (REV. 10-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER IT0013P	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>09/856886</b>	
INTERNATIONAL APPLICATION NO PCT/EP99/09207		INTERNATIONAL FILING DATE 24/11/1999		PRIORITY DATE CLAIMED 26/11/1998	
TITLE OF INVENTION PHARMACEUTICAL COMPOUNDS FOR THE INHIBITION OF HEPATITIS C VIRUS NS3 PROTEASE					
APPLICANT(S) FOR DO/EO/US ANTONELLO PESSI, PAOLA INGALLINELLA, ELISABETTA BIANCHI					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This is an express request to begin national examination procedures [35 U.S.C. 371(f)] at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(i).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made and the US was elected by the expiration of the 19th month from the earliest claimed priority date (PCT Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed [35 U.S.C. 371(c)(2)].</p> <p style="margin-left: 20px;">a. <input checked="" type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> has been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed [35 U.S.C. 371(c)(2)].</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 [35 U.S.C. 371(c)(3)].</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p style="margin-left: 20px;">d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 [35 U.S.C. 371(c)(3)].</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) [35 U.S.C. 371(c)(4)].</p> <p>10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 [35 U.S.C. 371(c)(5)].</p>					
Items 11 to 16 below concern other document(s) or information included:					
<p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.</p> <p style="margin-left: 20px;"><input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter</p> <p>16. <input type="checkbox"/> Other items or information:</p>					

**EXPRESS MAIL CERTIFICATE**  
DATE OF DEPOSIT MAY 25 2001  
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I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS  
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AN ENVELOPE ADDRESSED TO ASSISTANT COMMISSIONER  
FOR PATENTS, WASHINGTON, D.C. 20231  
MAILED BY Kathy K. Krawiec  
DATE 5-25-01

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/856886	PCT/EP99/09207	IT0013P

<p>17. <input checked="" type="checkbox"/> The following fees are submitted:</p> <p><b>BASIC NATIONAL FEE [37 CFR 1.492(a)(1)-(5)]:</b></p> <p>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee [37 CFR 1.445(a)(2)] paid to USPTO and International Search Report not prepared by the EPO or JPO . . . . . \$1,000.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO . . . . . \$860.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee [37 CFR 1.445(a)(2)] paid to USPTO . . . . . \$710.00</p> <p>International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) . . . . . \$690.00</p> <p>International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . . \$100.00</p> <p style="text-align: center;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT</b> =</p> <p>Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date [37 CFR 1.492(e)].</p> <table border="1" style="width:100%; border-collapse: collapse;"> <tr> <th style="width:25%;">Claims</th> <th style="width:25%;">Number Filed</th> <th style="width:25%;">Number Extra</th> <th style="width:25%;">Rate</th> </tr> <tr> <td>Total Claims</td> <td>21 - 20 =</td> <td>1</td> <td>X \$18.00</td> </tr> <tr> <td>Independent Claims</td> <td>1 - 3 =</td> <td>0</td> <td>X \$80.00</td> </tr> <tr> <td colspan="3">Multiple dependent claim(s) (if applicable)</td> <td>+ \$270.00</td> </tr> <tr> <td colspan="3"><b>TOTAL OF ABOVE CALCULATIONS</b></td> <td>= \$878.00</td> </tr> </table> <p><input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.</p> <p style="text-align: right;"><b>SUBTOTAL</b> = \$878.00</p> <p>Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date [37 CFR 1.492(f)].</p> <p style="text-align: right;"><b>TOTAL NATIONAL FEE</b> = \$878.00</p> <p>Fee for recording the enclosed assignment [37 CFR 1.21(h)]. The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property.</p> <p style="text-align: right;"><b>TOTAL FEES ENCLOSED</b> = \$878.00</p> <table border="1" style="width:100%; border-collapse: collapse;"> <tr> <td style="width:60%;"></td> <td style="width:40%; text-align: center;">Amount to be refunded</td> </tr> <tr> <td></td> <td style="text-align: center;">charged</td> </tr> </table>	Claims	Number Filed	Number Extra	Rate	Total Claims	21 - 20 =	1	X \$18.00	Independent Claims	1 - 3 =	0	X \$80.00	Multiple dependent claim(s) (if applicable)			+ \$270.00	<b>TOTAL OF ABOVE CALCULATIONS</b>			= \$878.00		Amount to be refunded		charged	<p style="text-align: center;"><b>CALCULATIONS</b></p> <p style="text-align: center;"><b>PTO USE ONLY</b></p>
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	Amount to be refunded																								
	charged																								

a. ☐ A check in the amount of \$ \_\_\_\_\_ to cover the above fees is enclosed.


b. ☒ Please charge my Deposit Account No. 13-2755 in the amount of \$878.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to the Deposit Account No. 13-2755. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive [37 CFR 1.137(a) or (b)] must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

MERCK & CO., INC.  
Patent Department, RY60-30  
P.O. Box 2000  
126 East Lincoln Avenue  
Rahway, New Jersey 07065-0970

  
 \_\_\_\_\_  
 SIGNATURE  
 \_\_\_\_\_  
 SHELTON O. HEBER  
 NAME  
 \_\_\_\_\_  
 38,179  
 REGISTRATION NUMBER

DATE: May 25, 2001

PHONE #: (732) 594-1958

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Pessi, et al.

Serial No.: Case No.: IT0013P

Filed:

For: PHARMACEUTICAL COMPOUNDS  
FOR THE INHIBITION OF HEPATITIS C  
VIRUS NS3 PROTEASEPRELIMINARY AMENDMENTAssistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Please amend the application filed herewith as provided below.

In the Claims:

Please cancel claim 13 without prejudice to future prosecution.

Please amend the claims by replacing and adding claims as follows:

3. (Amended Once) The compound of claim 1, a pharmaceutically acceptable salt or derivative thereof which is N-terminally acylated.

4. (Amended Once) A compound, salt or derivative according to claim 1 wherein A<sup>2</sup>B<sup>1</sup>-C<sup>2</sup>D<sup>1</sup> is a tetrapeptide of formula:

Pro-B<sup>1</sup>-C<sup>1</sup>-Leuwherein B<sup>1</sup> and C<sup>1</sup> are as defined in claim 1.

## EXPRESS MAIL CERTIFICATE

DATE OF DEPOSIT MAY 28 2001EXPRESS MAIL NO. EM 230307 696 US

I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS  
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FOR PATENTS, WASHINGTON, D.C. 20231.

MAILED BY Kathy K. LippincottDATE 5-28-01

5. (Amended Once) A compound, salt or derivative according to claim 4 wherein B' is selected from:  $\beta$ -cyclohexylalanine, phenylglycine, homophenylalanine, norleucine, leucine, methionine, norvaline and  $\beta$ -cyclopropylalanine.

7. (Amended Once) A compound, salt or derivative according to claim 5 wherein C' is selected from: aspartic acid, glutamic acid,  $\gamma$ -carboxyglutamic acid, glutamine, asparagine, hydroxyproline, N- $\beta$ -Aloc- diaminobutyric acid, thiazolylalanine, methionine sulfoxide, pyridylalanine and serine.

9. (Amended Once) A compound, salt or derivative according to claim 4, wherein the combination of amino acids B'C' is selected from:

Cha-Ser

Cha-Asp

Nle-Asp

Hof-Asp

Phg-Asp

Cha-Gln

Nle-Gln

Hof-Gln

Cha-Hyp

Nle-Hyp

Hof-Hyp

Nle-Ser.

10. (Amended Once) A compound, salt, or derivative according to claim 9 wherein Pep-OH is capable of binding HCV NS3 protease, in the absence of the C-terminal residues A'-B'-C'-D', and has an IC<sub>50</sub> below 100 $\mu$ M in an inhibition assay.

11. (Amended Once) A compound, salt, or derivative according to claim 9 wherein Pep is a hexa-, penta- or tetra-peptide having formula (II) below:

F-E-D-C-B-A

wherein: A is an amino acid or amino acid analogue having an aliphatic side chain of from 1 to 6 carbon atoms;

B is an amino acid or analogue having a non-polar, acidic, or polar but uncharged side group;

C is an amino acid or amino acid analogue having a non-polar or acidic side chain;

D is an amino acid or amino acid analogue having a hydrophobic side group;

E together with F may be absent, but if present is an amino acid or amino acid analogue having an acidic side chain, non-polar side chain or polar, but uncharged side chain, or is a dicarboxylic acid containing up to 6 carbon atoms and lacking the amino group of acidic amino acids;

and F may be absent (either by itself, or together with E) but when present is an amino acid or analogue having an acidic side chain or is a dicarboxylic acid containing up to 6 carbon atoms.

14. (Amended Once) A pharmaceutical composition comprising a compound, salt or derivative according to claim 1 and a pharmaceutically acceptable excipient, diluent or carrier.

15. (Amended Once) Use of a compound, salt or derivative according to claim 1 in the manufacture of a medicament for the treatment or prevention of hepatitis C or a related condition.

16. (Amended Once) A method of inhibiting HCV NS3 protease activity, and/or of treating or preventing hepatitis C or a related condition, comprising administering to a human or mammalian subject suffering from the condition a therapeutically or prophylactically effective amount of a composition according to claim 14.

17. (New) The composition of claim 14, wherein  
A' is proline,

B' is selected from the group consisting of:  $\beta$ -cyclohexylalanine, phenylglycine, homophenylalanine, norleucine, leucine, methionine, norvaline and  $\beta$ -cyclopropylalanine;

C' is selected from the group consisting of aspartic acid, glutamic acid,  $\gamma$ -carboxyglutamic acid, glutamine, asparagine, hydroxyproline, N- $\beta$ -Aloc- diaminobutyric acid, thiazolylalanine, methionine sulfoxide, pyridylalanine and serine,

D' is Leu,

or a pharmaceutically acceptable salt or derivative thereof.

18. (New) The composition of claim 17, wherein B' is either phenylglycine or  $\beta$ -cyclopropylalanine, or a pharmaceutically acceptable salt or derivative thereof.

19. (New) The composition of claim 17, wherein C' is aspartic acid, or a pharmaceutically acceptable salt or derivative thereof.

20. (New) The composition of claim 14, wherein

A' is proline,

D' is leucine,

B'C' is a combination selected from the group consisting of: Cha-Ser, Cha-Asp, Nle-Asp, Hof-Asp, Phg-Asp, Cha-Gln, Nle-Gln, Hof-Gln, Cha-Hyp, Nle-Hyp, Hof-Hyp, and Nle-Ser,

or a pharmaceutically acceptable salt or derivative thereof.

21. (New) The composition of claim 20, wherein Pep is a hexa-, penta- or tetra-peptide having formula (II) below:

F-E-D-C-B-A

A is selected from: cysteine, aminobutyric acid, di- and tri-fluoro aminobutyric acid, norvaline, allylglycine and alanine;

B is selected from: glutamic acid, aspartic acid, glycine, methyl glycine, 2-amino butyric acid, alanine, isoleucine, valine, leucine, cysteine, naphthylalanine and  $\beta$ -cyclohexylalanine;

C is selected from: glutamic acid, aspartic acid, glycine, methyl glycine, 2-amino butyric acid, alanine, isoleucine, valine, leucine, cysteine, naphthylalanine and  $\beta$ -cyclohexylalanine;

D is selected from: methionine, isoleucine, leucine, norleucine, valine, methylvaline, phenylglycine, diphenylalanine, tyrosine, thienylalanine, and chlorophenylalanine;

E is selected from: glutamic acid, aspartic acid, phenylalanine, diphenylalanine, isoleucine, valine, tyrosine, 4-nitrophenylalanine, glutaric acid and succinic acid;

and F is selected from: aspartic acid, glutamic acid, glutaric acid and succinic acid or a pharmaceutically acceptable salt or derivative thereof.

22. (New) A method of inhibiting HCV NS3 protease activity, and/or of treating or preventing hepatitis C or a related condition, comprising administering to a human or mammalian subject suffering from the condition a therapeutically or prophylactically effective amount of a compound according to claim 1.

Remarks

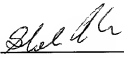
The present amendment cancels claim 13, amends claims 3, 4, 5, 7, 9, 10, 11, 14, 15, and 16, and adds claims 17, 18, 19, 20, 21, 22. The cancellation of claim 13 and the changes to the claims are without prejudice to future prosecution.

The changes to the claims are being made to eliminate the use of a multiple dependent claim format, to insert some new claims previously described using a multiple dependent claim format, and to correct typographically errors in claims 9 and 10. The changes to the claims do not narrow the claimed invention, but rather correspond to originally filed multiple dependent claims.

To save on filing costs, the full range of claims eliminated by removing multiple dependencies were not reintroduced. Applicant reserves the right to reintroduce such claims at a later time.

Accordingly the claims are in condition for allowance. Please charge deposit account 13-2755 for fees due in connection with this amendment. If any time extensions are needed for the timely filing of the present amendment, Applicants petition for such extensions and authorize the charging of deposit account 13-2755 for the appropriate fees.

Respectfully submitted,

By   
Sheldon O. Heber  
Reg. No. 38,179  
Attorney for Applicants

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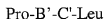


Version with Markings to Show Changes Made

Changes to the Claims:

3. (Amended Once) The compound of claim 1 [or claim 2], a pharmaceutically acceptable salt or derivative thereof which is N-terminally acylated.

4. (Amended Once) A compound, salt or derivative according to ~~[any one of claims]~~ claim 1 [to 3] wherein A'-B'-C'-D' is a tetrapeptide of formula:



wherein B' and C' are as defined in claim 1.

5. (Amended Once) A compound, salt or derivative according to ~~[any one of the previous claims]~~ claim 4 wherein B' is selected from:  $\beta$ -cyclohexylalanine, phenylglycine, homophenylalanine, norleucine, leucine, methionine, norvaline and  $\beta$ -cyclopropylalanine.

7. (Amended Once) A compound, salt or derivative according to ~~[any one of the previous claims]~~ claim 5 wherein C' is selected from: aspartic acid, glutamic acid,  $\gamma$ -carboxyglutamic acid, glutamine, asparagine, hydroxyproline, N- $\beta$ -Aloc- diaminobutyric acid, thiazolylalanine, methionine sulfoxide, pyridylalanine and serine.

9. (Amended Once) A compound, salt or derivative according to ~~[any one of the preceding claims]~~ claim 4, wherein the combination of amino acids B'C' is selected from:

Cha-Ser

Cha-Asp

Nle-Asp

Hof-Asp

Phg-Asp

Cha-Gln

Nle-Gln

Hof-Gln

Cha-Hyp

Nle-Hyp

Hof-Hyp

Nle-Ser<sub>2</sub>

10. (Amended Once) A compound, salt, or derivative according to [any-one-of-the preceding-claims] claim 9 wherein Pep-OH is capable of binding HCV NS3 protease, in the absence of the C-terminal residues A'-B'-C'-D', and has an IC<sub>50</sub> below 100μM in an inhibition assay.

11. (Amended Once) A compound, salt, or derivative according to [any-one-of-the preceding-claims] claim 9 wherein Pep is a hexa-, penta- or tetra-peptide having formula (II) below:



wherein: A is an amino acid or amino acid analogue having an aliphatic side chain of [form] from 1 to 6 carbon atoms;

B is an amino acid or analogue having a non-polar, acidic, or polar but uncharged side group;

C is an amino acid or amino acid analogue having a non-polar or acidic side chain;

D is an amino acid or amino acid analogue having a hydrophobic side group;

E together with F may be absent, but if present is an amino acid or amino acid analogue having an acidic side chain, non-polar side chain or polar, but uncharged side chain, or is a dicarboxylic acid containing up to 6 carbon atoms and lacking the amino group of acidic amino acids;

and F may be absent (either by itself, or together with E) but when present is an amino acid or analogue having an acidic side chain or is a dicarboxylic acid containing up to 6 carbon atoms.

14. (Amended Once) A pharmaceutical composition comprising a compound, salt or derivative according to ~~[any one of the preceding claims]~~ claim 1 and a pharmaceutically acceptable excipient, diluent or carrier.

15. (Amended Once) Use of a compound, salt or derivative according to ~~[any one of the preceding claims]~~ claim 1 in the manufacture of a medicament for the treatment or prevention of hepatitis C or a related condition.

16. (Amended Once) A method of inhibiting HCV NS3 protease activity, and/or of treating or preventing hepatitis C or a related condition, comprising administering to a human or mammalian subject suffering from the condition a therapeutically or prophylactically effective amount of a composition according to claim 14 ~~[, or of a compound of any one of claims 1 to 12].~~

PHARMACEUTICAL COMPOUNDS FOR THE INHIBITION  
OF HEPATITIS C VIRUS NS3 PROTEASE

Technical Field

- 5 This invention relates to compounds which can act as inhibitors of the hepatitis C virus (HCV) NS3 protease, to uses of such compounds and to their preparation.

Background Art

- 10 The hepatitis C virus (HCV) is the major causative agent of parenterally-transmitted and sporadic non-A, non-B hepatitis (NANB-H). Some 1% of the human population of the planet is believed to be affected. Infection by the virus can result in chronic hepatitis and cirrhosis of the liver, and may lead to  
15 hepatocellular carcinoma. Currently no vaccine nor established therapy exists, although partial success has been achieved in a minority of cases by treatment with recombinant interferon- $\alpha$ , either alone or in combination  
20 with ribavirin. There is therefore a pressing need for new and broadly-effective therapeutics.

- Several virally-encoded enzymes are putative targets for therapeutic intervention, including a metalloprotease  
25 (NS2-3), a serine protease (NS3), a helicase (NS3), and an RNA-dependent RNA polymerase (NS5B). The NS3 protease

is located in the N-terminal domain of the NS3 protein, and is considered a prime drug target since it is responsible for an intramolecular cleavage at the NS3/4A site and for downstream intermolecular processing at the NS4A/4B, NS4B/5A and NS5A/5B junctions.

Previous research has identified classes of peptides, in particular hexapeptides, showing degrees of activity in inhibiting the NS3 protease. The aim of the present invention is to provide further compounds which exhibit similar, and if possible improved, activity.

According to the nomenclature of Schechter & Berger (1967, Biochem. Biophys. Res. Commun. 27, 157-162) cleavage sites in substrates for the NS3 protease are designated P6-P5-P4-P3-P2-P1...P1'-P2'-P3'-P4'-, with each P representing an amino acid, and the scissile bond lying between P1 and P1'. Corresponding binding sites on the enzyme are indicated as S6-S5-S4-S3-S2-S1...S1'-S2'-S3'-S4'.

The present applicant has previously disclosed so called product inhibitors which are based on the P-region of the natural cleavage sites and which have been optimised to low nanomolar potency ((1998) Biochemistry 37: 8899-8905 and (1998) Biochemistry 37: 8906-8914). These inhibitors extract much of their binding energy

from the C-terminal carboxylate, the remaining interactions with NS3 being similar to the ones used by the natural substrates, including binding in the  $S_1$  pocket and the prominent electrostatic interaction of the P6-P5 acidic couple.

At variance with the P region, the P' region of the substrate, while being important for catalysis, does not influence significantly ground-state binding to the enzyme as expressed by the  $K_m$  value. In other words, binding energy released by the substrate interaction with the enzyme to form an initial non-covalent complex is essentially due to the interaction of the residues of the P region; the P' region residues contribute to a lesser extent to the binding energy. Accordingly, peptides based on the P' region of the natural substrates (spanning residues P<sub>1</sub>'-P<sub>10</sub>') do not inhibit NS3 to any significant extent. This notwithstanding, inspection of the crystal structure of NS3 with or without 4A (and more recently of the NMR structure of NS3) shows the presence of binding pockets in the S' region which might be exploited for the binding of active-site directed inhibitors. S'-binding ligands would therefore display a range of interactions with the enzyme different from the ones used by the substrate, and represent a novel class of NS3 inhibitors.

Landro et al in (1997) Biochemistry 36, 9340-9348 synthesized certain non-cleavable decapeptides based on the NS5A/5B cleavage site by substituting the P<sub>1</sub>' serine by a bulky cyclic aromatic (tetrahydroisoquinoline-3-carboxylic acid) or smaller cyclic alkyl compound (proline or pipecolinic acid). They then investigated the interaction of these decapeptides with the substrate binding site of NS3 either in the presence or absence of NS4A cofactor. By looking at the effect of truncation at either the P or P' side of the molecule they concluded that most of the binding energy of the decapeptide is due to interactions with NS3-NS4A complex on the P side of the molecule. Truncation on the P' side produced a relatively large effect in the presence of NS4A cofactor, but less when NS4A was absent. They concluded that the P4' substrate Tyr residue present in their molecules was in close proximity, or in direct contact with NS4A and that this residue contributes significantly to binding in the presence of NS4A.

The present inventors have developed inhibitors which are more powerful than those described by Landro et al because they have better binding on their P' side. In other words, the inhibitors take advantage of binding to the S' region in addition to binding to the S-region of NS3. By varying the P' amino acid residues, the present

inventors have shown that the binding energy which may be extracted from S'-region binding is substantial, since inhibitors with optimised and non-optimised P'-regions differ in potency > 1000-fold. Since no activity was present in any of the peptides corresponding to the isolated P'-region, optimisation of an S'-binding fragment was pursued in the context of non-cleavable decapeptides spanning P<sub>6</sub>-P<sub>4</sub>'.

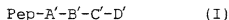
10           The inventors found that, by replacing Landro's P4' Tyr residue by leucine the effectiveness of the decapeptides as NS3 protease inhibitors could be enhanced. Although it had been previously shown that leucine in position P4' is better than tyrosine in a decapeptide substrate cleavable by NS3 (Urbani et al 15 (1997) J. Biol. Chem 272, 9204-9209), this is the first showing that the same applies to decapeptide inhibitors which are not cleaved under the influence of the enzyme. By optimising the P4' residue and then the P2'-P3' fragment 20 and using these together with an optimised P region the inventors have arrived at oligopeptides which show potency in the low nanomolar-subnanomolar range.

#### Disclosure of the Invention

25           According to a first aspect of the present invention there is provided a compound having the formula (I)



(written from N-terminus to C-terminus):



5            wherein "Pep" is a peptide or peptide analogue  
capable of binding to HCV NS3 protease; in particular, it  
is capable of binding in the S-region of the protease;

10           A' is proline which is optionally substituted, for  
instance with one to three substituent groups;

15           B' is an amino acid or amino acid analogue having a  
non polar side chain. Preferably, the side chain is an  
alkyl, aryl or aralkyl group containing 3 to 10,  
particularly 4 to 8 carbon atoms;

20           C' is an amino acid or amino acid analogue having a  
polar side group. Examples of polar side group may  
contain between 2 and 10, preferably 2 to 6 carbon atoms;

25           D' is leucine, or less preferably another amino acid  
with a non-polar aliphatic side chain, such as valine,  
isoleucine, norleucine or methionine. Alternatively, it  
is a short peptide or peptide analogue having one of  
these amino acids, especially leucine at its N terminus.  
The short peptide or peptide analogue may, for instance

comprise 2 to 6, preferably 2 to 4 amino acids or amino acid analogues.

As used herein, the term "amino acid analogue"  
5 includes organic compounds containing an amino and a carboxylic acid group, for instance arranged  $\alpha$ - to each other, and which do not necessarily occur in nature.

The Pep-A bond of the compound of formula (I) is  
10 substantially uncleavable by HCV NS3 protease. For instance, it is preferable that no cleavage be detectable using the assay described below under the heading "Substrate Assay".

15 Pharmaceutically acceptable salts of the compound of formula (I), as well as derivatives, such as esters are within the scope of the present invention.

Preferably, the compound of formula (I) is N-  
20 terminally acylated, especially acetylated, although other derivatives of the N-terminus are also possible, for instance N-terminal sulfoxide, sulphonamide, urethane or urea derivatives.

25 Preferably, the compound of formula (I) is C-terminally amidated. However, the C-terminus may be an

underivatized carboxylic acid group. Alternatively,  
other C-terminal groups may be present.

Assuming no substitution of the proline residue at A'  
5 is present, then a preferred C-terminal portion of the  
compound of formula I is:

Pro-B'-C'-Leu

10 possibly with a short C terminal extension at Leu.

Preferred examples of the amino acid, or analogue, B'  
for inclusion in compounds of the first aspect of the  
invention, include:

15  $\beta$ -cyclohexylalanine, phenylglycine,  
homophenylalanine and norleucine; other possibilities,  
though less preferred, are leucine, methionine,  
norvaline, and  $\beta$ -cyclopropylalanine. Of all these,  
20 cyclohexylalanine and phenyl glycine are most preferred.

Examples of the amino acid or analogue, C' include  
aspartic acid, glutamic acid,  $\gamma$ -carboxyglutamic acid,  
glutamine, asparagine, and hydroxyproline. Slightly less  
25 preferred are N- $\beta$ -Aloc-diaminobutyric acid,  
thiazolylalanine, methionine sulfoxide, pyridylalanine

and serine. Of all of these aspartic acid is most preferred.

The following combinations of amino acid residues at  
 5 B' and C' are preferred, of which the combination of cyclohexylalanine and aspartic acid is especially preferred.

TABLE 1

B'	C'
Cha	Ser
Cha	Asp
Nle	Asp
Hof	Asp
Phg	Asp
Cha	Gln
Nle	Gln
Hof	Gln
Cha	Hyp
Nle	Hyp
Hof	Hyp
Nle	Ser

Notes: Cha =  $\beta$ -cyclohexylalanine.

Nle = norleucine.

25 Phg = phenylglycine.

Hof = homophenylalanine.

Hyp = hydroxyproline.

When the residue D' is leucine (or other amino acid)

with a small peptide as C-terminal extension the peptide may be chosen by comparison with the corresponding P' portion of natural substrates.

- 5           The residues A', B', C' and D' may have D- or L- stereochemistry, although L-stereochemistry is, in general, preferred for each.

- 10           As regards the Pep part of the compound of formula (I) this is particularly preferably a peptide or peptide analogue capable of binding to HCV NS3 protease, even in the absence of the C-terminal residues A'-B'-C'-D', for instance when Pep carries just a carboxylic acid group at the C terminus. For example, when tested in the
- 15           inhibition assay described below the fragment Pep-OH preferably has an IC<sub>50</sub> below 100µM, e.g. below 20µM, particularly below 10µM and, optimally, of less than 1µM. Preferably, Pep is a hexa-, penta- or tetra peptide having formula II below:

20



- wherein: A is an amino acid or amino acid analogue having a relatively small (C<sub>1</sub>-C<sub>6</sub>) aliphatic side chain.
- 25           Possible choices for this group include cysteine, aminobutyric acid (Abu) (including di- and tri-fluoro

Abu), norvaline, allylglycine and alanine, any of which may be N-methylated. Of these, cysteine and the fluorinated aminobutyric acids are preferred choices for A.

5

B is an amino acid or analogue having a non-polar or acidic side chain. Some amino acids having polar but uncharged side groups may also be suitable. Examples of suitable amino acids include glutamic and aspartic acid, glycine and methyl glycine, 2-amino butyric acid, alanine, isoleucine, valine, leucine, cysteine, naphthylalanine and  $\beta$ -cyclohexylalanine. Of these, cyclohexylalanine is particularly preferred.

10

15

C is an amino acid or amino acid analogue having a non-polar or acidic side chain. For instance, the examples of such amino acids given above for B apply also to C. In this case isoleucine and glutamic acid are particularly preferred.

20

D is usually an amino acid or amino acid analogue having a hydrophobic side group such as methionine, isoleucine, leucine, norleucine, valine, methyl valine, phenylglycine or, diphenylalanine. Among these leucine and, particularly, diphenylalanine are preferred. Some polar amino acids which include hydrophobic portions,

25

such as tyrosine, thienylalanine, and chlorophenylalanine may be suitable.

E together with F may be absent, but if present is generally an amino acid or amino acid analogue having an acidic side chain. Preferred examples are glutamic and aspartic acid, with the former being preferred. E may, alternatively, be an amino acid or analogue having a non-polar, or polar but uncharged side chain. Of the non-polar amino acids, phenylalanine, diphenylalanine, isoleucine and valine are preferred, especially the D-enantiomers. Among the polar amino acids suitable examples are tyrosine and 4-nitrophenylalanine. Alternatively, where F is absent (see below), E may be a dicarboxylic acid containing up to 6 carbon atoms and lacking the amino group of acidic amino acids. Suitable examples are glutaric and succinic acid.

F may be absent (either by itself, or together with E), but when present is an amino acid or analogue having an acidic side chain. Aspartic acid is preferred, although glutamic acid is another possibility. Like E, F may also be a dicarboxylic acid containing up to 6 carbon atoms, and lacking the amino group of acidic amino acids. Examples are glutaric and succinic acid.

Of residues E and F preferably at least E is present. Particularly preferably both are present.

5 The amino acids and analogues A-F may be either L- or D- enantiomers though L- is generally preferred for all residues. In some cases it may be beneficial for one or other of the residues to be D- while the rest are L-. In particular it may be advantageous for E to be D-glu.

10 Preferred examples of the peptide "Pep" are listed below in Tables 2 and 3 together with their IC<sub>50</sub> values when unextended at the C-terminus. Except for the compounds having a succinyl residue at the N-terminus, all compounds tested were N-acetylated at the N-terminus.



TABLE 2

Exp No.	Sequence	IC <sub>50</sub> (μM)
5	1 Asp Glu Met Glu Glu Cys	1.0
	2 Asp Glu Met Glu Glu D-Cys	4.0
	3 Asp Glu Met Glu Glu Abu	5.8
	4 Met Glu Glu Cys	150.0
	5 Glu Met Glu Glu Cys	21.0
10	6 Glu Asp Val Val Cys Cys	5.3
	7 Glu Asp Val Val Abu Cys	2.8
	8 Asp Glu Val Val Cys Cys	2.1
	9 Glu Asp Val Val Gly Cys	20.0
	10 Asp Glu Met Glu Glu Alg	12.0
15	11 Glu Asp Val Val MGly Cys	21.0
	12 Glu Asp MVal Val Abu Cys	1.3
	13 GluS Met Glu Glu Cys	1.3
	14 AsGlu Met Glu Glu Cys	0.6
	15 Asp Glu Met Glu Leu Cys	1.1
20	16 Asp Glu Met Glu Cha Cys	0.3
	17 Asp Glu Met Glu Nap Cys	0.8
	18 AspS Val Val Abu Cys	4.6
	19 Asp Glu Met Glu Glu Cys(Me)	16.7
	20 Asp Glu Val Glu Cha Cys	0.33

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21	Asp Glu Ile Glu Cha Cys	0.12
22	Asp Glu Tyr Glu Cha Cys	0.24
23	Asp Glu Phe Glu Cha Cys	0.42
24	Asp Glu Leu Glu Cha Cys	0.12
25	Asp Glu Cha Glu Cha Cys	0.14
26	Asp Glu Nle Glu Cha Cys	0.22
27	Asp Glu Tha Glu Cha Cys	0.87
28	Asp Glu FCI Glu Cha Cys	0.3
29	Asp Glu Phg Glu Cha Cys	0.12
30	Asp Glu Dif Glu Cha D-Cys	3.4
31	Glu Dif Glu Cha Cys	1.4
32	Dif Glu Cha Cys	30.0
33	Asp MGlu Leu Glu Cha Cys	1.0
34	Asp Glu Dif Glu Cha DHala	7.1
35	Asp Glu Met Glu Glu Cpc	9.0
36	Glu Dif Ile Cha Cys	2.5
37	Dif Ile Cha Cys	100.0
38	Asp Glu Met Glu Glu CnAla	19.0
39	Asp Glu Leu Glu Cha Abu	1.6

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40	Asp Glu Leu Glu Cha Val	4.0
41	Asp Glu Leu Glu Cha Nva	1.3
42	Asp-Asp-Leu-Glu-Cha-Cys	0.290
43	Asp-Pno-Leu-Glu-Cha-Cys	0.240
44	Asp-Tyr-Leu-Glu-Cha-Cys	0.135
45	Asp- (D) Phe-Leu-Glu-Cha-Cys	0.820
46	Asp- (D) Tyr-Leu-Glu-Cha-Cys	0.680
47	Asp- (D) Val-Leu-Glu-Cha-Cys	0.470
48	Asp- (D) Ile-Leu-Glu-Cha-Cys	0.330
49	Asp- (D) Dif-Leu-Glu-Cha-Cys	0,276
50	Asp- (D) Asp-Leu-Glu-Cha-Cys	0,122
51	Asp-Glu-Dap (N-b-Dns) -Glu-Cha-Cys	0,4

Particularly preferred examples of Pep, together  
with their IC<sub>50</sub>s (in  $\mu$ M) are set out below in Table 3 are:

TABLE 3

5

Most preferred:

10	1	Asp Glu Dif Glu Cha Cys	0.05
	2	Asp Glu Leu Val Cha Cys	0.08
	3	Asp Glu Leu Ile Cha Cys	0.06
	4	Asp Glu Dif Ile Cha Cys	0.06
	5	Asp-Gla-Leu-Glu-Cha-Cys	0.055
15	6	Asp- (D) Glu-Leu-Glu-Cha-Cys	0.045
	7	Asp- (D) Gla-Leu-Ile-Cha-Cys	0.0015
	8	Glu-Leu-Glu-Cha-Cys	1.3
	9	(D) Glu-Leu-Glu-Cha-Cys- (Pro-Cha-Asp-Leu)	0.080*
	10	Succinyl Glu-Leu-Ile-Cha-Cys	
20	11	Succinyl (D) Glu-Leu-Glu-Cha-Cys- (Pro-Cha- Asp-Leu)	0.0040*
	12	Asp- (D) Glu-Leu-Ile-Cha-Cys	
	13	Asp- (D) Glu-Leu-Ile-Cha-Cys- (Pro-Cha-Asp- Leu)	<0.0002*

\* Tested only as decapeptides

In these compounds:

	Alg	=	allylglycine.
5	MGly	=	methylglycine.
	MVal	=	methylvaline.
	Abu	=	2-aminobutyric acid.
	GluS	=	N-succinylglutamic acid.
	AsGlu	=	Glutamic acid having N-terminal
10			acetylsulphonamide.
	Cha	=	$\beta$ -cyclohexylalanine.
	Nap	=	naphthylalanine.
	AspS	=	N-succinylaspartic acid.
	Nle	=	norleucine.
15	Dif	=	3,3-diphenylalanine.
	Tha	=	2-thienylalanine.
	FCI	=	4-chlorophenylalanine.
	Phg	=	phenylglycine.
	CysMe	=	S-methylcysteine.

	Cys (ACS)	=	Cysteine with C-terminal acylsulphonamide.
	DHAla	=	dehydroalanine.
5	Cpc	=	1-amino-1-cyclopentane carboxylic acid.
	CnAla	=	cyanoalanine.
	MGlu	=	N-methylglutamic acid.
	Fno	=	4-nitrophenylalanine.
	Gla	=	$\gamma$ -carboxyglutamic acid.
10	Dap	=	$\beta$ -diaminopropionic acid.
	Dns	=	dansyl (5-dimethylamino-1- naphthalene-sulfonyl).

Examples of compounds of the present invention may  
15 be effective as inhibitors of NS3 protease at micromolar  
or nanomolar levels. Preferably, the  $IC_{50}$ , as measured in  
the assay described below is less than 100nM,  
particularly preferably less than 20nM and, optimally,  
less than 5nM.

According to a second aspect, the present invention provides a compound, salt or derivative according to the first aspect, for use in any therapeutic method, preferably for use in inhibiting the HCV NS3 protease, and/or for use in treating or preventing hepatitis C or a related condition. By "related condition" is meant a condition which is or can be caused, directly or indirectly, by the hepatitis C virus, or with which the HCV is in any way associated.

10

According to a third aspect the present invention provides the use of a compound or derivative according to the first aspect in the manufacture of a medicament for the treatment or prevention of hepatitis C or a related condition.

15

A fourth aspect of the invention provides a pharmaceutical composition which includes one or more compounds or derivatives according to the first aspect.

The composition may also include pharmaceutically acceptable adjuvants such as carriers, buffers, stabilisers and other excipients. It may additionally include other therapeutically active agents, in particular those of use in treating or preventing hepatitis C or related conditions.

The pharmaceutical composition may be in any suitable form, depending on the intended method of administration. It may for example be in the form of a tablet, capsule or liquid for oral administration, or of a solution or suspension for administration parenterally.

According to a fifth aspect of the invention, there is provided a method of inhibiting HCV NS3 protease activity, and/or of treating or preventing hepatitis C or a related condition, the method involving administering to a human or animal (preferably mammalian) subject suffering from the condition a therapeutically or



prophylactically effective amount of a composition according to the fourth aspect of the invention, or of a compound or derivative according to the first aspect.

"Effective amount" means an amount sufficient to cause a benefit to the subject or at least to cause a change in the subject's condition.

The dosage rate at which the compound, derivative or composition is administered will depend on the nature of the subject, the nature and severity of the condition, the administration method used, etc. Appropriate values can be selected by the trained medical practitioner. Preferred daily doses of the compounds are likely to be of the order of about 1 to 100 mg. The compound, derivative or composition may be administered alone or in combination with other treatments, either simultaneously or sequentially. It may be administered by any suitable route, including orally, intravenously, cutaneously, subcutaneously, etc. Intravenous administration is preferred. It may be administered directly to a suitable

site or in a manner in which it targets a particular site, such as a certain type of cell - suitable targeting methods are already known.

5           A sixth aspect of the invention provides a method of preparation of a pharmaceutical composition, involving admixing one or more compounds or derivatives according to the first aspect of the invention with one or more pharmaceutically acceptable adjuvants, and/or with one or  
10 more other therapeutically or prophylactically active agents.

          According to a seventh aspect of the invention there is provided a method of producing the compounds of  
15 formula I. These compounds may be generated wholly or partly by chemical synthesis beginning from individual, preferably protected, amino acids or oligopeptides and using known peptide synthesis methods.

Modes for Carrying Out the Invention

Embodiments of the invention are exemplified below by way of illustration only.

5 EXAMPLES(1) Synthesis

The synthesis of one of the compounds of the present invention is described below. Other compounds may be synthesized by an analogous method.

Synthesis of Ac-Asp-(D)Glu-Leu-Ile-Cha-Cys-Pro-Cha-Asp-Leu-Pro-Tyr-Lys(N<sup>ε</sup>-Ac)-NH<sub>2</sub>

15 The synthesis was performed on solid phase by the continuous-flow Fmoc-polyamide method (Atherton, E. and Sheppard, R. C. (1989) Solid phase peptide synthesis. A practical approach, IRL Press, Oxford.). The resin used was Tentagel<sup>®</sup> derivatised with a modified Rink amide

linker p-[(R,S)- $\alpha$ -[1-(9H-Fluoren-9-yl)-methoxyformamido]-  
2,4-dimethoxybenzyl]-phenoxyacetic acid (Rink, H. (1987)  
*Tetrahedron Lett.* **28**, 3787-3789; Bernatowicz, M. S.,  
Daniels, S. B. and Koster, H. (1989) *Tetrahedron Lett.*  
5 **30**, 4645-4667). All the coupling reactions were performed  
for 30 min with 5-fold excess of activated amino acid  
over the resin free amino groups, using Fmoc-amino  
acid/PyBOP/HOBt/DIEA (1:1:1:2) activation; double  
coupling was used for the cysteine residue. At the end of  
10 the assembly, the dry peptide-resin was treated with  
trifluoroacetic acid/water/triisopropylsilane  
(92.5:5:2.5) for 1.5h at room temperature; the resin was  
filtered out and the peptide precipitated with cold  
methyl t-Bu ether; the precipitate was redissolved in 50%  
15 water/acetonitrile containing 0.1%TFA and lyophilised.

Purification to >98% homogeneity was achieved  
through preparative HPLC on a Waters RCM (C-18) column  
(100 X 25 mm, 15mm) using as eluents (A) 0.1%  
20 trifluoroacetic acid in water and (B) 0.1%

trifluoroacetic acid in acetonitrile. The gradient used was 40%B isocratic for 5 min, then 40-60%B over 20 min, flow rate 30 ml/min; the fractions were analysed by HPLC (column: Beckman Ultrasphere, C-18, 25 X 4.6 mm, 5min; gradient: 35-65%B in 20 min, same eluents as the preparative run, flow 1ml/min) and those containing the pure material were pooled and lyophilised (yield=50%). The Mass spectrum was acquired on a Perkin-Elmer API-100 spectrometer: MS= 1695.03 (calc.) 1694.6 (found).

## (2) Inhibition Assay

The ability of the compounds to inhibit NS3 protease was evaluated using an NS3/4A complex comprising the NS3 protease domain and a modified form of the NS4A peptide, Pep 4AK [KKKGSVVIVGRIILSGR(NH<sub>2</sub>)]. As substrate, a substrate peptide 4AB [DEMEECASHLPYK] based on the sequence of the NS4A/NS4B cleavage site of the HCV polyprotein, was used.

Cleavage assays were performed in 57  $\mu$ l 50 mM Hepes  
pH7.5, 1 % CHAPS, 15 % glycerol, 10 mM DTT (buffer A), to  
which 3  $\mu$ l substrate peptide were added. As protease co-  
factor a peptide spanning the central hydrophobic core  
(residues 21-34) of the NS4A protein, Pep4AK  
[KKKGSVVIVGRIILSGR(NH<sub>2</sub>)] was used. Buffer solutions  
containing 80  $\mu$ M Pep4AK were preincubated for 10 minutes  
with 10-200 nM protease and reactions were started by  
addition of substrate. Six duplicate data points at  
different substrate concentrations were used to calculate  
kinetic parameters. Incubation times were chosen in  
order to obtain <7% substrate conversion and reactions  
were stopped by addition of 40  $\mu$ l 1 % TFA. Cleavage of  
peptide substrates was determined by HPLC using a Merck-  
Hitachi chromatograph equipped with an autosampler. 80  
 $\mu$ l samples were injected on a Lichrospher C18 reversed  
phase cartridge column (4 x 74mm, 5  $\mu$ m, Merck) and  
fragments were separated using a 10-40 % acetonitrile  
gradient a 5%/min using a flow rate of 2.5ml/min. Peak  
detection was accomplished by monitoring both the

absorbance at 220nm and tyrosine fluorescence ( $\lambda_{ex}$  = 260 nm,  $\lambda_{em}$  = 305nm). Cleavage products were quantitated by integration of chromatograms with respect to appropriate standards. Kinetic parameters were calculated from nonlinear least-squares fit of initial rates as a function of substrate concentration with the help of a Kaleidagraph software, assuming Michaelis-Menten kinetics.

- 10  $K_i$  values of peptide inhibitors were calculated from substrate titration experiments performed in the presence of increasing amounts of inhibitor. Experimental data sets were simultaneously fitted to eq.1 using a multicurve fit macro with the help of a Sigmaplot software:

$$V = (V_{max}S) / (K_m(1+K_i/I)+S); \quad (\text{eq.1})$$

Alternatively,  $K_i$  values were derived from IC50

values, calculated using a four-parameter logistic function, according to eq.2:

$$IC_{50} = (1+S/K_m)K_i \quad (eq.2)$$

5

The table below sets out the  $IC_{50}$  values for a variety of peptides tested in this assay and establishes that several optimised compounds of the present invention are active at nanomolar or subnanomolar levels. All the compounds tested - except for compound 26 which has a succinyl residue at the N-terminus- were tested as their N-acetyl derivatives.

Some of these compounds are the most potent in vitro inhibitors of HCV protease described to date. They are reversible, non covalent inhibitors which do not contain an electrophilic ("serine-trap") moiety in the molecule. They bind to both the S and S' region of the enzyme, and this makes them suitable for developing competition



binding assays, since they would be competitive with compounds binding to either the S or the S' region of the enzyme.

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Table 4

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Ex. No	Sequence	IC <sub>50</sub> (nM)
1	Glu Asp Val Val Abu Cys Pro Nie Ser Tyr	8500
2	Glu Asp Val Val Abu Cys (Me)Ala Nie Ser Tyr	3500
3	Asp Glu Dif Ile Cha Abu Ala Ser His Leu	29000
4	Asp Glu Dif Ile Cha Abu (Me)Ala Ser His Leu	29000
5	Asp Glu Dif Ile Cha (Me)Abu Ala Ser His Leu	8000
6	Asp Glu Dif Ile Cha (Me)Abu (Me)Ala Ser His Leu	3800
7	Asp (D)Glu Dif Ile Cha (Me)Abu (Me)Ala Ser His Leu	3100
8	Asp (D)Glu Leu Ile Cha Abu (Me)Ala Ser His Leu	5000
9	Asp Glu Dif Ile Cha Cys Pro Nie Ser Tyr	876
10	Glu Dif Ile Cha Cys Pro Nie Ser Leu	64
11	Asp Glu Dif Ile Cha Cys Pro Cha Ser Leu	23
12	Asp Glu Dif Ile Cha Cys Pro Cha Asp Leu	1.3
13	Asp Glu Dif Ile Cha Cys Pro Phg Asp Leu	7
14	Asp Glu Dif Ile Cha Cys Pro Nie Asp Leu	1.8
15	Asp Glu Dif Ile Cha Cys Pro Hof Asp Leu	1.8
16	Asp Glu Dif Ile Cha Cys Pro Cha Gln Leu	14
17	Asp Glu Dif Ile Cha Cys Pro Nie Gln Leu	32
18	Asp Glu Dif Ile Cha Cys Pro Hof Gln Leu	18

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19	Asp Glu Dif Ile Cha Cys Pro Cha Hyp Leu	11
20	Asp Glu Dif Ile Cha Cys Pro Nle Hyp Leu	26
21	Asp Glu Dif Ile Cha Cys Pro Hof Hyp Leu	15
22	Asp (D)Glu Leu Ile Cha Cys Pro Nle Ser Leu	10
23	Asp Glu Dif Ile Cha Cys Pro Cha Asp Leu PYK(Ac)	0.85
24	Asp (D)Glu Leu Ile Cha Cys Pro Cha Asp Leu PYK(Ac)	< 0.2
25	Asp (D)Glu Leu Ile Cha Cys Pro Cha Asp Leu	< 0.2
26	Suc-(D)Glu Leu Ile Cha Cys Pro Cha Asp Leu	4
27	Asp (D)Glu Leu Glu Cha Cys Pro Cha Asp Leu	0.63
28	(D)Glu Leu Glu Cha Cys Pro Cha Asp Leu	80
29	Asp (D)Glu Leu Glu Cha Ala Pro Cha Asp Leu	17
30	Asp (D)Glu Leu Ile Cha Cys Pro Nle Ser Leu	10

10

## Abbreviations used in Table I:

Abu = aminobutyric acid

Cha =  $\beta$ -cyclohexylalanine

Hof = homophenylalanine

5 Hyp = hydroxyproline

Lys(Ac) or K(Ac) = Ne-Acetyl-Lysine

Nle = norleucine

Phg = phenylglycine

Sta = statine [(3S,4S)-4-amino-3-hydroxy-6-

10 methylheptanoic acid]

Dif = 3,3-diphenylalanine

Suc=succinyl

N-methylation is indicated as (Me) preceding the three-  
letter code of the amino acid

15 PYK = proline-tyrosine-lysine

(3) Substrate Assay

In order to determine whether or not an inhibitor  
molecule was a substrate for HCV NS3 protease a modified

version of the cleavage assay described above was employed using, as before, an NS3/4A complex comprising the NS3 protease domain and a modified form of the NS4A peptide, Pep4AK [KKKGSVVIVGRIILSGR(NH<sub>2</sub>)].

5

1μM of the enzyme complex was incubated for 16hrs in the presence of 10μM inhibitor as a candidate substrate peptide. Assays were performed in 57μl 50 mM Hepes pH7.5, 1% CHAPS, 15% glycerol, 10 mM DTT.

10

After this time HPLC was used to separate any peptides resulting from cleavage and separated cleavage products detected.

15

Samples were analysed by HPLC on a Beckman 0.46 x 25 cm C18 reversed phase column equilibrated in 95% solvent A (0.1% TFA in H<sub>2</sub>O) and 5% solvent B (0.1% TFA in acetonitrile) at a flow rate of 1 ml/min. Samples were eluted from this column with a linear gradient from 5% to

90% of B in 45 minutes. Peak detection was accomplished by monitoring absorbance at 220 nm.

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[illegible]

5                      Pep-A'-B'-C'-D'                      (I)

10           A' is a proline residue which is optionally  
substituted;

C' is an amino acid or amino acid analogue having a  
15 polar side chain; and

D' is selected from leucine, other amino acids or amino acid analogues having a non-polar aliphatic side chain, and peptides of 2 to 6 amino acids having leucine or other amino acid or amino acid analogue with a non-polar aliphatic side chain as N-terminal residue;

2. The compound of claim 1, a pharmaceutically  
25 acceptable salt or derivative thereof which is C-

terminally amidated.

3. The compound of claim 1 or claim 2, a  
pharmaceutically acceptable salt or derivative thereof  
5 which is N-terminally acylated.

4. A compound, salt or derivative according to any one  
of claims 1 to 3 wherein A'-B'-C'-D' is a tetrapeptide of  
formula:

10 Pro-B'-C'-Leu

wherein B' and C' are as defined in claim 1.

5. A compound, salt or derivative according to any one  
of the previous claims wherein B' is selected from:  $\beta$ -  
15 cyclohexylalanine, phenylglycine, homophenylalanine,  
norleucine, leucine, methionine, norvaline and  $\beta$ -  
cyclopropylalanine.

6. A compound, salt or derivative according to claim 5  
20 wherein B' is selected from cyclohexylalanine and  
phenylglycine.

7. A compound, salt or derivative according to any one  
of the previous claims wherein C' is selected from:  
25 aspartic acid, glutamic acid,  $\gamma$ -carboxyglutamic acid,



glutamine, asparagine, hydroxyproline, N- $\beta$ -Aloc-diaminobutyric acid, thiazolylalanine, methionine sulfoxide, pyridylalanine and serine.

5      8. A compound, salt or derivative according to claim 7 wherein C' is aspartic acid.

9. A compound, salt or derivative according to any one of the preceding claims, wherein the combination of amino  
10      acids B'C' is selected from:

Cha-Ser

Cha-Asp

Nle-Asp

Hof-Asp

15      Phg-Asp

Cha-Gln

Nle-Gln

Hof-Gln

Cha-Hyp

20      Nle-Hyp

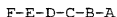
Hof-Hyp

Nle-Ser

10. A compound, salt, or derivative according to any one  
25      of the preceding claims wherein Pep-OH is capable of

binding HCV NS3 protease, in the absence of the C-terminal residues A'-B'-C'-D', and has an  $IC_{50}$  below 100 $\mu$ M in an inhibition assay.

- 5 11. A compound, salt, or derivative according to any one of the preceding claims wherein Pep is a hexa-, penta- or tetra-peptide having formula (II) below:



- 10 wherein: A is an amino acid or amino acid analogue having an aliphatic side chain of form 1 to 6 carbon atoms;

B is an amino acid or analogue having a non-polar, acidic, or polar but uncharged side group;

- 15 C is an amino acid or amino acid analogue having a non-polar or acidic side chain;

D is an amino acid or amino acid analogue having a hydrophobic side group;

- 20 E together with F may be absent, but if present is an amino acid or amino acid analogue having an acidic side chain, non-polar side chain or polar, but uncharged side chain, or is a dicarboxylic acid containing up to 6 carbon atoms and lacking the amino group of acidic amino acids;

- 25 and F may be absent (either by itself, or together with E) but when present is an amino acid or analogue

having an acidic side chain or is a dicarboxylic acid containing up to 6 carbon atoms.

12. A compound according to claim 11 wherein:

5           A is selected from: cysteine, aminobutyric acid, di- and tri-fluoro aminobutyric acid, norvaline, allylglycine and alanine;

10           B is selected from: glutamic acid, aspartic acid, glycine, methyl glycine, 2-amino butyric acid, alanine, isoleucine, valine, leucine, cysteine, naphthylalanine and  $\beta$ -cyclohexylalanine;

15           C is selected from: glutamic acid, aspartic acid, glycine, methyl glycine, 2-amino butyric acid, alanine, isoleucine, valine, leucine, cysteine, naphthylalanine and  $\beta$ -cyclohexylalanine;

            D is selected from: methionine, isoleucine, leucine, norleucine, valine, methylvaline, phenylglycine, diphenylalanine, tyrosine, thienylalanine, and chlorophenylalanine;

20           E is selected from: glutamic acid, aspartic acid, phenylalanine, diphenylalanine, isoleucine, valine, tyrosine, 4-nitrophenylalanine, glutaric acid and succinic acid;

25           and F is selected from: aspartic acid, glutamic acid, glutaric acid and succinic acid.

13. A compound, salt, or derivative, according to any one of the preceding claims for use in therapy.

5 14. A pharmaceutical composition comprising a compound, salt or derivative according to any one of the preceding claims and a pharmaceutically acceptable excipient, diluent or carrier.

10 15. Use of a compound, salt or derivative according to any one of the preceding claims in the manufacture of a medicament for the treatment or prevention of hepatitis C or a related condition.

15 16. A method of inhibiting HCV NS3 protease activity, and/or of treating or preventing hepatitis C or a related condition, comprising administering to a human or mammalian subject suffering from the condition a therapeutically or prophylactically effective amount of a composition according to claim 14, or of a compound of  
20 any one of claims 1 to 12.

**COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY**  
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER

IT0013

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Pharmaceutical compounds for the inhibition of hepatitis C virus NS3 protease

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application

Serial No.

on

and was amended

on

(if applicable).

☒ was filed as PCT international application

Number PCT/EP99/09207

on 24 November 1999

and was amended under PCT Article 19

on

(if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

**PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:**

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
Great Britain	9825946.8	26 November 1998	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

**PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:**

U.S. APPLICATIONS			STATUS (Check one)		
U S APPLICATION NUMBER	U S FILING DATE		PATENTED	PENDING	ABANDONED
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO	PCT FILING DATE	US SERIAL NUMBERS ASSIGNED (if any)			
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration number) S. O. Heber - 38,179 J. L. Tribble - 32,633

Send Correspondence to <u>Merck &amp; Co., Inc., 126 E. Lincoln Avenue, Rahway, New Jersey 07065, U.S.A.</u>			Direct Telephone Calls to (name and telephone number) <u>S. O. Heber (732)594-1958</u>	
2	FULL NAME OF INVENTOR	<u>FAMILY NAME</u> <u>Bianchi</u>	FIRST GIVEN NAME	<u>Elisabetta</u>
0	RESIDENCE & CITIZENSHIP	<u>CITY</u> <u>Rome</u>	STATE OR FOREIGN COUNTRY	<u>Italy</u>
1	POST OFFICE ADDRESS	<u>POST OFFICE ADDRESS</u> <u>IRBM, Via Pontina KM 30.600</u>	CITY	<u>Pomezia (Rome)</u>
2	FULL NAME OF INVENTOR	<u>FAMILY NAME</u> <u>Ingallinella</u>	FIRST GIVEN NAME	<u>Paolo</u>
0	RESIDENCE & CITIZENSHIP	<u>CITY</u> <u>Naples</u>	STATE OR FOREIGN COUNTRY	<u>Italy</u>
2	POST OFFICE ADDRESS	<u>POST OFFICE ADDRESS</u> <u>IRBM, Via Pontina KM 30.600</u>	CITY	<u>Pomezia (Rome)</u>
2	FULL NAME OF INVENTOR	<u>FAMILY NAME</u> <u>Pessi</u>	FIRST GIVEN NAME	<u>Antonello</u>
0	RESIDENCE & CITIZENSHIP	<u>CITY</u> <u>Rome</u>	STATE OR FOREIGN COUNTRY	<u>Italy</u>
3	POST OFFICE ADDRESS	<u>POST OFFICE ADDRESS</u> <u>IRBM, Via Pontina KM 30.600</u>	CITY	<u>Pomezia (Rome)</u>
			STATE & ZIP CODE/COUNTRY	<u>I-00040, Italy</u>

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 <u>Elisabetta Bianchi</u>	SIGNATURE OF INVENTOR 202 <u>Paolo Ingallinella</u>	SIGNATURE OF INVENTOR 203 <u>Antonello Pessi</u>
DATE <u>May 31st 2001</u>	DATE <u>May 31st 2001</u>	DATE <u>May 31st 2001</u>